Review Quiroz et al-

This is a manuscript reporting on several years of experimentation with restoration of the foundational scleractinian Acropora palmata on the Caribbean coast of Mexico. The manuscript needs some more information in the introduction to add context for the benefit of the readers and, to enhance the significance of the results and improve the discussion. For example, a short paragraph summarizing why Ap went almost extinct across its entire geographic distribution, and what factors had kept its recovery slow, and the short and long-term results of other Caribbean restoration attempts, why they failed after a few years, why none of them reflects current areas of natural recover of Ap populations. There are many localities where Ap has and is naturally recovering over wide areas much better than any restoration projects.

It is also important to discuss the spatial scales of the restoration projects in comparison with the extensive coral reef systems and some discussion on what are the major differences in the approach used in this project compared to the many other attempts at Ap restoration would enhance the ms.

A couple of paragraphs in the discussion seem to belong in the methods section.

Main conclusions should be clearly stated and maybe put in bullets.

Below I included several changes and suggestions to consider in the revised version

Line 21 - When you said “lack of recovery after disease outbreaks” are you referring to a particular locality? There is ample evidence of population recovery in many localities after the 1983-84 die-off.

Most acroporid restoration programs, however, have been unsuccessful in the long term. They usually collapse (after a few years of showing good growth) due to a new environmental or biological impact.

Introduction

Line 54 – replace “key” for “ foundational”.

Line 56 – “disease outbreaks in the late 1970’s” there was one disease outbreak, white band disease, that was observed in the early 1980’s across the geographical distribution of acroporids, that wiped out >95% of the populations.

Line 56 – “low larval recruitment” – do you have evidence for this? Or are you referring to low survival of juveniles. Recruitment is usually high, we just do not observe the single primary polyps.

Line 56 – what threatens the viability of the species is the continuous disruption of their environment by anthropogenic activities in combination with increased hurricane frequency and intensity, thermal anomalies (bleaching), and recurrent WBD local outbreaks after the initial die-off.

Line 68 – There are more recent references on impact of diseases like SCTLD, that have contributed significantly to the degradation.

Line 73 – “protected” elaborate, is protected from wave action or because is an endangered species? Add reference to the later if this is what you mean.

Lines 78 to 82 – there is no context to this paragraph, that stars by mentioning what the results were. Need to add some information of the project, questions, goals/objectives, hypotheses, and brief

summary of methods, before the results. The reader does not know what the project is about at the end of the introduction as it is.

The introduction needs more information on natural recovery patterns of both acroporid species in different localities, and address the question of why some localities do better than others? And then, include the restoration activities as a small contribution to head-start local populations and enhance sexual reproduction to increase genetic variability. Some key references (and more there in) are:

Weil E, Hernandez AE, Bruckner AW, Ortiz AL, Nemeth M, Ruiz H. 2002. Distribution and status of acroporid (scleractinia) coral populations in Puerto Rico. Proceedings of the Caribbean Workshop: Potential Application of the US Endangered Species Act (ESA) as a Conservation Strategy. NOAA-NMFS and NCORE_RSMAS. U. of Miami 71_92.

Lucas MQ, Weil E. 2015. Recovery in Acropora cervicornis and abundance of A. prolifera off La Parguera. Puerto Rico: Marine Biodiversity.

Quinn NJ, Kojis BL. 2006. Evaluating the potential of natural reproduction and artificial techniques to increase Acropora cervicornis populations at Discovery Bay, Jamaica. Review Biological Tropical 54:105_116.

Weil E, Hammerman NM, Becicka RL, Cruz-Motta JJ. 2020. Growth dynamics in Acropora cervicornis and A. prolifera in southwest Puerto Rico. PeerJ 8:e8435 http://doi.org/10.7717/peerj.8435

Methods

Line 96 – Culture water samples ……..

Line 96 – how did you estimate fertilization success? Explain.

Line 97- What proportion of the water was replaced daily? (25%, 50%,100% ??)

Line 99 – Explain how, where, for how long, and why were the plugs “pre-conditioned”. Be consistent, use either “pre-conditioned plugs” or “pre-conditioned substrate”.

Line 127 - …. Depth. Each ….. (period)

Line 146 – delete “the”..

Line 170 _ delete “the” ..

Survival and growth

Growth rates seem to be very low in the early stages, any idea why?

Line 210 - … 100%...

Lines 261-262- This is a risky statement if you do not define what is considered as “recruitment”. I do not think they know what the settlement rates are, which is different from recruitment rates, which is

usually estimated from small juvenile colonies with a few polyps. This statement probably reflects juvenile survivorship mainly.

Lines 263-272- This whole paragraph belongs in the methods and results.

Lines 276-280 – same as above??

Line 286 – “ecological restoration” ??? what does this mean? This is artificial or assisted “restoration” of a few colonies to impacted reefs areas, and represent the time scale of the observations, with potential for recovery of impacted populations, and increasing genetic diversity. This is not “permanent”, the next bleaching event or epizootic could obliterate the restored colonies.

The discussion needs some context to natural recovery of acroporids, and if the effort and investment ($$$) in restoration programs is worth it

I think authors should expand (and bullet) the overall and key biological and ecological conclusions.

Long-term survival, growth, and reproduction of *Acropora palmata* sexual recruits outplanted onto two Mexican Caribbean reefs (#81550)

First submission

Guidance from your Editor

Please submit by **2 Feb 2023** for the benefit of the authors (and your token reward) .

Structure and Criteria
Please read the 'Structure and Criteria' page for general guidance.

Author notes
Have you read the author notes on the guidance page?

Raw data check
Review the raw data.

Image check
Check that figures and images have not been inappropriately manipulated.

Privacy reminder: If uploading an annotated PDF, remove identifiable information to remain anonymous.

Files

Download and review all files from the materials page.

6 Figure file(s)
2 Table file(s)
1 Raw data file(s)

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Structure your review

The review form is divided into 5 sections. Please consider these when composing your review:

1. **BASIC REPORTING**
2. **EXPERIMENTAL DESIGN**
3. **VALIDITY OF THE FINDINGS**
4. General comments
5. Confidential notes to the editor

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Use these criteria points to structure your review. The full detailed editorial criteria is on your guidance page.

BASIC REPORTING

- Clear, unambiguous, professional English language used throughout.
- Intro & background to show context. Literature well referenced & relevant.
- Structure conforms to PeerJ standards, discipline norm, or improved for clarity.
- Figures are relevant, high quality, well labelled & described.
- Raw data supplied (see PeerJ policy).

EXPERIMENTAL DESIGN

- Original primary research within Scope of the journal.
- Research question well defined, relevant & meaningful. It is stated how the research fills an identified knowledge gap.
- Rigorous investigation performed to a high technical & ethical standard.
- Methods described with sufficient detail & information to replicate.

VALIDITY OF THE FINDINGS

- Impact and novelty not assessed. *Meaningful* replication encouraged where rationale & benefit to literature is clearly stated.
- All underlying data have been provided; they are robust, statistically sound, & controlled.
- Conclusions are well stated, linked to original research question & limited to supporting results.

Standout reviewing tips

The best reviewers use these techniques

Tip	*Example*
Support criticisms with evidence from the text or from other sources	*Smith et al (J of Methodology, 2005, V3, pp 123) have shown that the analysis you use in Lines 241-250 is not the most appropriate for this situation. Please explain why you used this method.*
Give specific suggestions on how to improve the manuscript	*Your introduction needs more detail. I suggest that you improve the description at lines 57- 86 to provide more justification for your study (specifically, you should expand upon the knowledge gap being filled).*
Comment on language and grammar issues	*The English language should be improved to ensure that an international audience can clearly understand your text. Some examples where the language could be improved include lines 23, 77, 121, 128 – the current phrasing makes comprehension difficult. I suggest you have a colleague who is proficient in English and familiar with the subject matter review your manuscript, or contact a professional editing service.*
Organize by importance of the issues, and number your points	*1. Your most important issue* *2. The next most important item* *3. …* *4. The least important points*
Please provide constructive criticism, and avoid personal opinions	*I thank you for providing the raw data, however your supplemental files need more descriptive metadata identifiers to be useful to future readers. Although your results are compelling, the data analysis should be improved in the following ways: AA, BB, CC*
Comment on strengths (as well as weaknesses) of the manuscript	*I commend the authors for their extensive data set, compiled over many years of detailed fieldwork. In addition, the manuscript is clearly written in professional, unambiguous language. If there is a weakness, it is in the statistical analysis (as I have noted above) which should be improved upon before Acceptance.*

Long-term survival, growth, and reproduction of *Acropora palmata* sexual recruits outplanted onto two Mexican Caribbean reefs

Sandra Mendoza Quiroz [1], **Aurora U. Beltrán-Torres** [2], **Daniela Muñoz Villareal** [3], **Raúl Tecalco Rentería** [1], **Victoria Grosso Becerra** [4], **Anastazia T Banaszak** [Corresp. 4]

[1] SECORE International, Miami, Florida, United States of America

[2] Jardín Botánico, El Colegio de la Frontera Sur, Puerto Morelos, Quintana Roo, Mexico

[3] Universidad Jorge Tadeo Lozano, Santa Marta, Magdalena, Colombia

[4] Unidad Académica de Sistemas Arrecifales, Universidad Nacional Autónoma de México, Puerto Morelos, Quintana Roo, Mexico

Corresponding Author: Anastazia T Banaszak
Email address: banaszak@cmarl.unam.mx

Background. *Acropora palmata* is a key, endangered Caribbean reef-building coral species. The lack of recovery after disease outbreaks and low recruitment has led to widespread use of fragmentation to restore populations. An alternative method of restoration is the production of recruits via assisted sexual reproduction to restotre genetic diversity. **Methods.** In 2011 and 2012, *A. palmata* sexual recruits were cultured, settled and raised in an *ex-situ* nursery. In 2014, the two cohorts were moved to an *in-situ* nursery. In 2015, a subset of the two cohorts (29 colonies) were outplanted onto Cuevones Reef. Growth and survival of these colonies were monitored periodically and compared to colonies that remained in the *in-situ* nursery. In 2019, samples were collected and analyzed for fertility and fecundity. A further 14 colonies from these same cohorts were outplanted in 2020 onto Picudas Reef and monitored during the following spawning seasons. In 2022, spawning was observed and gametes collected. The resultant sexual recruits (F2 generation) settled, were maintained in *ex-situ*, flow-through aquaria and monitored for growth and survival. **Results.** Both cohorts of settlers obeyed a Type III survival curve with high initial mortality and low later-stage mortality. The colonies had 100% survival while in the *in-situ* nursery and for an additional nine years after outplanting onto Cuevones Reef. Their growth rates increased three-fold after outplanting when compared to those in the *ex-situ* and *in-situ* nurseries. At Cuevones Reef, 53% of the colonies were fertile and fecundity was 5.61 ± 1.91 (average ± S.D.) oocytes and 3.04 ± 0.26 (average ± S.D.) spermaries per polyp. The colonies outplanted onto Picudas Reef had 100% survival two years after planting. In 2022, spawning of three of the colonies at Picudas Reef was recorded. Gametes from two of the colonies were collected and crossed, with 15% fertilization success. Spermatozoa from wild colonies were also added increasing

fertilization success to 95%. The larvae produced followed normal development and settled onto artificial concrete substrates that had been previously conditioned in the ocean. Within two weeks symbiont uptake was visible. As of September 2022, the sexual recruits are maintained in culture in an aquarium system and periodically monitored for survival and growth. **Discussion.** The long-term survival of outplanted *Acropora palmata* colonies produced via assisted sexual reproduction was shown to be high when outplanted onto reefs after an initial *ex-situ* husbandry period of 1.5 years (Cohort 2012) and 2.5 years (Cohort 2011). Growth rates of the colonies were higher when outplanted under natural reef conditions than in *ex-situ* or *in-situ* nurseries. Fertility and fecundity were confirmed in both cohorts, thus they are actively reproducing and contributing to the genetic diversity and establishment of self-sustaining sexually-reproducing populations of *Acropora palmata* in the Mexican Caribbean.

LONG-TERM SURVIVAL, GROWTH, AND REPRODUCTION OF *ACROPORA PALMATA* SEXUAL RECRUITS OUTPLANTED ONTO MEXICAN CARIBBEAN REEFS

Sandra Mendoza Quiroz[1], Aurora U. Beltrán Torres[2], Daniela Muñoz Villareal[3], Raúl Tecalco Rentería[1], María Victoria Grosso-Becerra[4], Anastazia T. Banaszak[4]

[1] SECORE International, Miami, Florida, United States of America.
[2] El Colegio de la Frontera Sur, Unidad Chetumal, Chetumal, Quintana Roo, Mexico.
[3] Programa de Biología Marina, Universidad de Bogotá Jorge Tadeo Lozano, Santa Marta, Colombia.
[4] Unidad Académica de Sistemas Arrecifales, Universidad Nacional Autónoma de México, Puerto Morelos, Quintana Roo, México.

Corresponding Author:
Anastazia T. Banaszak
Prol. Av. Niños Héroes, Puerto Morelos, Quintana Roo, 77580, México
Email address: banaszak@cmarl.unam.mx

Abstract

Background. *Acropora palmata* is a key, endangered Caribbean reef-building coral species. The lack of recovery after disease outbreaks and low recruitment has led to widespread use of fragmentation to restore populations. An alternative method of restoration is the production of recruits via assisted sexual reproduction to restotre genetic diversity. **Methods.** In 2011 and 2012, *A. palmata* sexual recruits were cultured, settled and raised in an *ex-situ* nursery. In 2014, the two cohorts were moved to an *in-situ* nursery. In 2015, a subset of the two cohorts (29 colonies) were outplanted onto Cuevones Reef. Growth and survival of these colonies were monitored periodically and compared to colonies that remained in the *in-situ* nursery. In 2019, samples were collected and analyzed for fertility and fecundity. A further 14 colonies from these same cohorts were outplanted in 2020 onto Picudas Reef and monitored during the following spawning seasons. In 2022, spawning was observed and gametes collected. The resultant sexual recruits (F2 generation) settled, were maintained in *ex-situ*, flow-through aquaria and monitored for growth and survival. **Results.** Both cohorts of settlers obeyed a Type III survival curve with high initial mortality and low later-stage mortality. The colonies had 100% survival while in the *in-situ* nursery and for an additional nine years after outplanting onto Cuevones Reef. Their growth rates increased three-fold after outplanting when compared to those in the *ex-situ* and *in-situ* nurseries. At Cuevones Reef, 53% of the colonies were fertile and fecundity was 5.61 ± 1.91 (average ± S.D.) oocytes and 3.04 ± 0.26 (average ± S.D.) spermaries per polyp. The colonies outplanted onto Picudas Reef had 100% survival two years after planting. In 2022, spawning of three of the colonies at Picudas Reef was recorded. Gametes from two of the colonies were collected and crossed, with 15% fertilization success. Spermatozoa from wild colonies were also

added increasing fertilization success to 95%. The larvae produced followed normal development and settled onto artificial concrete substrates that had been previously conditioned in the ocean. Within two weeks symbiont uptake was visible. As of September 2022, the sexual recruits are maintained in culture in an aquarium system and periodically monitored for survival and growth. **Discussion.** The long-term survival of outplanted *Acropora palmata* colonies produced via assisted sexual reproduction was shown to be high when outplanted onto reefs after an initial *ex-situ* husbandry period of 1.5 years (Cohort 2012) and 2.5 years (Cohort 2011). Growth rates of the colonies were higher when outplanted under natural reef conditions than in *ex-situ* or *in-situ* nurseries. Fertility and fecundity were confirmed in both cohorts, thus they are actively reproducing and contributing to the genetic diversity and establishment of self-sustaining sexually-reproducing populations of *Acropora palmata* in the Mexican Caribbean.

Introduction

Acropora palmata is a key Caribbean reef-building coral and currently listed as a critically endangered species on the IUCN Red List. The lack of recovery after disease outbreaks in the late 1970s and low larval recruitment threaten the viability of this species, which has led to widespread intervention using coral fragments to replenish depleted populations. The use of sexual recruits is also important for population recovery to maintain or potentially increase genetic diversity.

Several cases have been recorded where colonies of *Acropora* species from the Indo-Pacific region such as *A. tenius* and *A. millepora* have been cultured and have reached sexual maturity after three and nine years of age (Iwao et al., 2010; Baria *et al.,* 2012; Guest et al., 2014; Baria-Rodriguez *et al.,* 2019). In the Caribbean, the spawning of *Acropora palmata* colonies reared from their larval stage to being reproductively active at four years of age was first reported by Chamberland et al., (2016). *Acropora palmata* is a hermaphrodite, housing oocytes and spermaries in the same polyp. Spawning of oocytes and sperm packaged together in gamete bundles occurs in summer months after the full moon, typically in August.

Reefs along that Mexican Caribbean have shown signs of degradation due to hurricanes, storms, disease, and bleaching (Jordán-Dahlgren and Rodríguez-Martínez, 1998), as well as a marked increase in coastal development in the area (Rioja-Nieto and Álvarez-Filip, 2019). One example of such a reef is Picudas Reef located in the Puerto Morelos Reef National Park. Reefs can also be damaged by ships. For example, in December 1997, the Norwegian cruise ship "Leeward" impacted the reef crest of Cuevones Reef, which was composed primarily of the protected, reef-building species *Acropora palmata* (Padilla Souza *et al*., 2018). This grounding affected a total area of 504 m^2 reducing the live coral cover from 14% to 1%. By 2016 coral cover had recovered to 4.5% principally composed of *Porites astreoides* and *Agaricia agaricites*, but there were no signs of natural recruitment of reef-building species such as *Acropora palmata* (Padilla Souza *et al*., 2018).

Here we show that the colonies outplanted to both Cuevones Reef and Picudas Reef have reached sexual maturity and have the potential to naturally contribute to population replenishment, which is the end objective of all restoration programs. We have also successfully produced an F2

generation of *Acropora palmata* recruits from the colonies produced in our culture facility and outplanted to Picudas Reef.

Materials & Methods

Gamete collection, larval culture, and settlement in *ex-situ* nurseries

Acropora palmata gamete bundles (containing eggs and sperm) were collected from wild parent colonies at La Bocana Reef (20° 59' 18.74" N, 86° 47' 26.66" W) in the Puerto Morelos Reef National Park, Mexican Caribbean. Spawning occurred 5 nights after full moon in August 2011 and September 2012. Collecting nets with containers at the top were deployed on presumed genetically distinct colonies by divers, respectively. The collecting containers were lidded and taken to the dive boat, where the gamete bundles from all parents were combined for fertilization in filtered and UV-C sterilized sea water (FSW). The gametes were gently mixed for 30 seconds at five-minute intervals to assist the fertilization process and transported to the laboratory. After two hours, the mixture was rinsed to remove excess sperm and predators, using fat-separating pitchers, and placed in 100 L coolers containing FSW maintained at 28 °C until settlement. After four hours, samples were examined under a dissecting microscope to estimate fertilization success. Daily water changes were performed to eliminate unfertilized eggs, metabolized lipids, organic matter, and mucus to avoid bacterial proliferation (Banaszak et al. 2018).

Once the larvae began to swim, previously conditioned substrates in the form of plugs were placed into the culture bins. The substrate plugs were manufactured using a mixture of Portland Type II cement and beach sand (1:1) in the form of plugs and conditioned in the sea for two months prior to larval settlement. On retrieval from the sea, the substrates were scrubbed to remove sediment and overgrowing algae before their placement in the culture bins. Initial settlement counts were made at five to eight days post-settlement. The settlers were inoculated at 10 days post-settlement with symbiotic dinoflagellates that were freshly isolated from *A. palmata* fragments using FSW and an air brush (Tytler and Spencer 1983, Baird *et al.,* 2006). During the inoculation period, the water level in the larval culture bins was reduced and a concentration of at least $1x10^6$ symbiont cells/mL was added. After 16 h, a partial water change was made, and three days later infection was inspected using a dissecting microscope and fluorescent light.

The settlers were maintained in outdoor aquaria at UNAM or the Xcaret Aquarium facility (Fig. 1). Both *ex situ* nurseries were outfitted with flowing sea water at an overall average temperature of 29 ± 0.6 °C (average ± SD) and peak noon irradiance ranging from 300 to 600 µmol m^{-2} s^{-1}. Temperatures were measured using HOBO water temperature data loggers (HOBO UA-001-64 Pendant Temp) and irradiance was measured using a LI-COR LI-192 underwater quantum sensor connected to a LI-COR 1400 data logger. The settled recruits were fed daily, initially using commercially available feed (Kent Marine, USA) according to the recommendations of the manufacturer, and later with rotifers and fresh *Artemia* nauplii. The aquarium walls and floors were cleaned twice a week to remove filamentous algae, whereas the substrates were carefully cleaned with the naked eye to avoid settlers on a weekly basis to reduce algal overgrowth. Detailed monitoring of the settlers was begun at the third month post-settlement (Table 1). For the 2012

cohort, colony area and number of polyps were obtained from photographs analysed with ImageJ software.

Transfer to and monitoring of *in-situ* nurseries

In April 2014 a subset of colonies from the two cohorts (2011 and 2012) were transferred from the two *ex-situ* nurseries to two *in-situ* nurseries (Fig. 1), one located in the Puerto Morelos Reef National Park at 5 m depth and the second in Las Ruinas, Playa del Carmen at 3.8 m depth, each site received 12 (Cohort 2011) and 18 recruits (Cohort 2012) from the *ex-situ* nurseries. The *in-situ* nurseries consisted of 45 cm high vertical supports made from 2.5 cm diameter PVC tubes incorporated into cement plates that measured 50 cm x 50 cm x 20 cm (L x W x H). Each support had a PVC connector at its free end which was used to mount the coral colony via a compatible connector (Johnson *et al*., 2011). The average daily temperature in Las Ruinas nursery, as measured by a HOBO water temperature data logger (HOBO UA-001-64 Pendant Temp) was 28 ± 0.3 °C with solar noon irradiance ranging between 230 and 710 µmol m^{-2} s^{-1} as measured by an Odyssey Waterproof Photosynthetic Active Radiation Logger. The average daily temperature in the Puerto Morelos nursery, as measured by a HOBO water temperature data logger (HOBO UA-001-64 Pendant Temp) was 29± 0.5 °C (average ± SD) with solar noon irradiance ranging between 450 and 600 µmol m^{-2} s^{-1} as measured by an Odyssey Waterproof Photosynthetic Active Radiation Logger. Survival and growth were determined at various intervals. For growth measurements, the size of the colony, as determined by maximum length, width, and height from the base, was recorded using a vernier caliper.

Outplanting onto Cuevones Reef

In February 2015, 29 of the surviving colonies from both 2011 and 2012 cohorts were harvested from both *in-situ* nurseries were outplanted to Cuevones Reef (21º 09' 4.14" N; 86º 44' 26.7" W) (Fig. 1) in the Isla Mujeres, Punta Nizuc and Punta Cancun National Marine Park at 10 m deep. The overall temperature averaged 29 ± 0.5 °C and average peak irradiance between 300 and 400 µmol m^{-2} s^{-1}. Each colony was attached to the reef using a mixture of cement and sand. The colonies were monitored every three months for survival and growth as described above. In August 2019, tissue samples were collected from the base of the edge of 10 colonies of 2011 cohort and 5 colonies of 2012, placed in ziploc bags with seawater, and transported to the laboratory. The samples were fixed in 10% formaldehyde in FSW for 24 hours at 4 °C, then rinsed with FSW. Then, al samples were decalcified by progressively increasing the concentration of hydrochloric acid (HCl, buffered with EDTA, Sodium Tartrate and Potassium) from 1 to 8 %. The carbonate-free tissue was preserved in 70% Ethanol. Between 10 and 14 polyps were dissected from each colony to document fertility (% of polyps with oocytes, Van Veghel and Kahmann, 1994), fecundity (number of oocytes per polyp (Kojis and Quinn 1981) and spermaries per polyp), polyp density, gonadal index (number of oocytes per cm^2) and oocyte diameter in selected polyps. Survival and growth were determined at various intervals. For growth measurements, the size of the colony, as determined by maximum length, width, and height from the base, was recorded. Data are presented as means (± standard deviation).

Outplanting onto Picudas Reef

In September 2020, two colonies from the 2011 cohort and 14 colonies from the 2012 cohort were outplanted onto Picudas Reef in the Puerto Morelos Reef National Park (Fig.1). Picudas Reef is 3 to 5 meters depth, with an overall average temperature of 29± 0.2 °C (average ± SD) °C and a peak irradiance between 400 and 550 µmol m^{-2} s^{-1}. The colonies were attached to the reef using a mixture of cement and sand. The survival, growth and spawning of all 16 colonies were monitored in the summer of the following two years. The benthic cover of the reef was obtained following the AGRRA method (Lang *et al*., 2012) the one month and 1.6 years after outplant. In August 2022, gametes from these F1 colonies were collected and cross-fertilized 85 min after spawning using 2 mL of gamete bundles from each colony. Separately, an extra cross was made 110 min after spawning with 1 mL of gamete bundles from an unrelated 'wild' colony and 30 mL of the sperm batch from the first cross of juvenile colonies. Embryos were cultured and settled as described above. The settled recruits were placed and are maintained in the UNAM *ex situ* nursery to date.

Results

F1 Fertilization and settlement success in *ex-situ* conditions

Gamete bundles were collected from two of the 10 colonies that spawned in 2011 and all four colonies that spawned in 2012. Fertilization success was 90% in 2011 and 99% in 2012. Approximately 2,000 recruits from the 2011 cohort and around 1,000 recruits from the 2012 cohort were inoculated with symbionts and raised in aquaria maintained under natural sunlight. During the first three months post-settlement, survival of the settlers was high after which there is a decrease to 31% at 4 months and 0.3% at 18 months for the 2011 cohort (Table 1). Survival of the 2012 cohort was 38% at 9 months and 12% at 18 months. Mortality was due to overgrowth by filamentous algae, failure to take up symbionts despite multiple inoculation attempts or competition between the coral recruits themselves. In some cases, two or more coral recruits would fuse to form one larger colony. A linear correlation was found between polyp number and colony area during the first year of growth (Fig. 2A, $R^2 = 0.99$). During the first year of growth, the number of polyps ($R^2 = 0.96$) and colony area ($R^2 = 0.94$) increased exponentially over time in *ex-situ* culture (Fig. 2B). Growth rates in the *ex-situ* nursery were 2.5 mm (± 0.7) per month for the 2012 cohort (Fig. 3).

Survival and growth in the *in-situ* nursery and after outplanting

The survival of the 60 juvenile colonies placed in the *in-situ* nursery was 100% for both cohorts during the 10-month period (Table 1). Twenty-nine colonies were outplanted onto Cuevones Reef, 16 were outplanted to Picudas Reef and 15 colonies remained in the *in-situ* nursery. Growth rates just prior to outplanting onto the reef were 1.9 mm (± 0.5) per month, which was slightly lower but not significantly different, to the growth rates recorded in colonies maintained in the *ex-situ* nursery during the same period (Fig. 3).

Three and a half years after the 29 colonies were outplanted onto Cuevones Reef, survival of both cohorts was 100%. Growth rates increased to 5.6 mm (± 1.8) per month, whereas the growth rates of the 15 colonies from the same cohort that remained in the *in-situ* nursery were 1.9 mm (± 0.5) per month (Fig. 3).

Prior to outplanting onto Picudas Reef, macroalgal cover was approximately 34% with 23% of turf algae, 23% crustose coralline algal cover and 3% of live corals. One and a half years after the outplanting the macroalgal cover decreased to 21% (Data not shown), with increase to 27% of turf algae, 27% crustose coralline algal cover and 5% of live corals. Of the 16 colonies that were outplanted onto Picudas Reef, survival was 100 at 2.5 years after outplanting. In the same period the range of growth rates was wide, from -260 mm to 350 mm per month. The negative growth is due to the partial damage of the colonies due to branch breakage from the impact of hurricanes Delta and Zeta that affected the Puerto Morelos Reef National Park in 2020 and the regrowth of the colonies (Fig. 4).

Reproductive parameters of outplanted colonies: Cuevones Reef

Based on the dissections of the polyps from the 15 colonies of sexual origin (Fig. 5A, 5B) that were sampled at Cuevones Reef, eight (53%) contained gonads. Of the 96 polyps that were dissected from the eight colonies with gonads, 76% contained oocytes and/or spermaries (Fig. 5C, 5D). Fecundity was 4.03 ± 2.7 (n = 387) oocytes per polyp and there were 2.30 ± 1.5 (n = 221) spermaries per polyp. The gonadal index was 63.63 ± 43.35 oocytes cm^{-2} (n = 8) and 35.03 ± 19.39 spermaries cm^{-2} (n = 8). The mean maximum diameter of the oocytes was 769 µm ± 141 (n = 42) and the mean diameter of the spermaries was 1.52 mm ± 0.423 (n = 14).

Fertilization success and settlement of F2 generation: Picudas Reef

In August 2022, spawning was recorded in 1 colony from the 2011 cohort and 3 colonies from the 2012 cohort (Fig. 6A, 6B, Table 2) and 18 other nearby colonies, 8 wild and 10 colonies of asexual origin, three nights after full moon at Picudas Reef. Two mL of gamete bundles were collected from each of two colonies of the 2012 cohort that spawned. Cross fertilization of these two colonies yielded low fertilization success at 15%. However, when their sperm were crossed separately with bundle gametes from a wild colony fertilization success increased to 95%. The embryos and larvae produced in the second cross were maintained in *ex-situ* aquaria with a settlement yield of 2.4% at 13 days after settlement (Fig. 6C). The settled larvae were successfully inoculated with symbionts (Fig. 6D) and raised in aquaria maintained under natural solar conditions. Survival at three months was 1.76%. The average diameter of the settlers was 1.2 ±0.3 mm (n = 19) and the average area was 1.49 ± 0.19 cm^2 (n = 19).

Discussion

In this study, we not only show that colonies of the coral species *Acropora palmata* of sexual origin, produced by crossing wild-caught gametes, reared in the laboratory and outplanted onto coral reefs, are reproductively viable, but they also can generate viable F2 offspring. Thus, the goal

of ecological restoration, to produce self-sustaining breeding populations of this critically endangered species is achievable.

Overall, the spawning pattern and behavior of both generations of offspring is congruent with wild populations. Colonies reared in the laboratory, even after maintenance for extended periods in *ex-situ* nurseries before outplanting onto coral reefs, liberated gametes within the known spawning window for this species and on the same night as their wild counterparts. Coinciding in spawning time and date makes it highly probable that fertilization between the laboratory-produced and natural colonies can occur; in fact, we showed that such a combination results in fertilization success of 95%, at least in the laboratory setting.

The outplanted colonies from both cohorts (7 and 8 years old) were shown to be fertile, producing oocytes and spermaries (presence or absence of oocytes and spermaries). The size of the oocytes is large at 769 μm ± 141 in diameter, which is similar to that of nine species of *Acropora* from the Great Barrier Reef, which ranged from 601 to 728 μm in diameter (Wallace 1985). The fecundity of the outplanted (number of oocytes per polyp) was 5.61 ± 1.91 and falls within the values reported for four species of *Acropora* from the Great Barrier Reef that ranged from 4.67 (± 0.60 SE) to 6.52 (± 0.84 SE) (Pratchett et al., 2019).

The performance of larvae and settlers was similar to that of other generations raised in our ex-situ larval culture facility (Miller et al. 2021). While corals spawn large numbers of gametes, the survivorship rate immediately after and in the early phases of the life cycles follows a type III survival curve. For most mass spawning coral species in the Caribbean, natural recruitment has failed over the last few decades (Hughes and Tanner 2000; Williams et al 2008). For example, in Cuevones Reef in the Cancun National Park, no natural recruitment of *A. palmata* was recorded, even twenty years after a ship grounding impacted the reef crest (Padilla-Souza et al. 2018). Therefore, to improve the settlement and survival success during the early phases of the *A. palmata* life cycle in our facility, we applied a series of protocols to optimize culture conditions. This intensive care of the settlers included weekly cleaning to reduce algal overgrowth, a primary source of early-stage coral recruit mortality. Applying these protocols resulted in the production of 70 colonies, of which 29 were outplanted to Cuevones Reef in the West Coast of Isla Mujeres, Punta Cancún and Punta Nizuc National Park and 16 to Picudas Reef in the Puerto Morelos Reef National Park. The rest of the surviving colonies were housed in an *in-situ* nursery or put on exhibition in a public aquarium. After outplanting onto the reef, the growth rates of the colonies increased approximately three-fold, when compared with their growth rates in either *ex-situ* or *in-situ* nurseries. Therefore, intensive care does result in the production of colonies that can thrive in the reef environment.

The outplanted colonies reached sexual maturity in both Cuevones Reef and Picudas Reef. We successfully collected gametes from the two colonies outplanted onto Picudas Reef and crossed them. Fertilization success was low at 15% indicating that these may be siblings, as both are colonies from the same cohort, due the initial fertilization took place with gametes from 4 parents. Once we added gametes from a wild colony the fertilization success increased to 95%. Therefore, assisted fertilization may result in the production of siblings, and these offspring should

be well spaced and preferably interspersed with wild (unrelated) colonies to improve fertilization potential and, importantly, reduce the chances of inbreeding. If wild colonies are not available, then we suggest that cohorts from different generations and from as many collection sites as possible be intermixed.

Overall, our results show that ecological restoration of corals can be achieved to not only reestablish the structural function of creating habitat, but also to enhance reproductive function in their natural environment

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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365

Figure 1

Timeline of the phases described in this study for two cohorts of *Acropora palmata* produced via assisted sexual reproduction.

The transfers of *Acropora palmata* colonies from two *ex-situ* nurseries (UNAM and Xcaret) to two *in-situ* nurseries (Puerto Morelos and Las Ruinas) and then to two reefs (Cuevones Reef and Picudas Reef) are shown. The ages of two cohorts of recruits are shown in blue (2011 cohort) and red (2012 cohort) in rows at the top and the corresponding colored arrows (with N = number of recruits) depict the transfer to the next phase. Samples for fecundity analysis were collected from Cuevones Reef and spawning observations and gamete sampling were undertaken at Picudas Reef. Note: The Las Ruinas *in-situ* nursery was decommissioned in 2015 when colonies were outplanted to Cuevones Reef; the remaining recruits were transferred to the Puerto Morelos nursery (dashed black arrow).

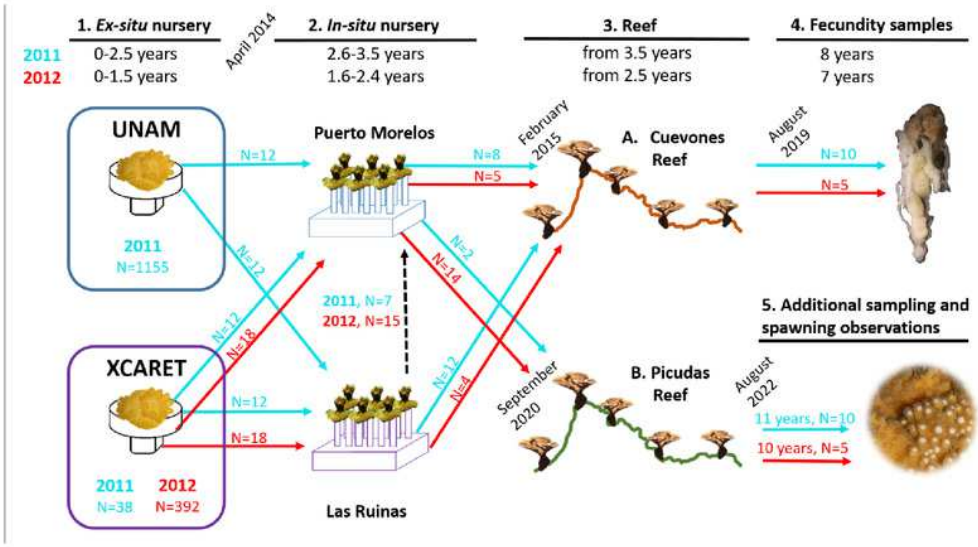
1. Ex-situ nursery
2. In-situ nursery
3. Reef
4. Fecundity samples
2011
2012
0-2.5 years
0-1.5 years
April 2014
2.6-3.5 years
1.6-2.4 years
from 3.5 years
from 2.5 years
8 years
7 years
UNAM
2011
N=1155
XCARET
2011
N=38
2012
N=392
Puerto Morelos
Las Ruinas
N=12
N=18
2011, N=7
2012, N=15
N=8
N=5
N=2
N=14
N=4
February 2015
A. Cuevones Reef
B. Picudas Reef
September 2020
August 2019
N=10
August 2022
11 years, N=10
10 years, N=5
5. Additional sampling and spawning observations

Figure 2

Growth of *Acropora palmata* recruits in *ex-situ* culture during 12 months after settlement.

(A) Linear correlation between growth (polyp number) and colony area ($R^2 = 0.9564$, $y = 0.7359x^{1.8849}$). (B) Polyp number (triangles) and colony area (circles) as an exponential function of recruit age in *ex-situ* culture ($R^2 = 0.9345$, $y = 0.0975x^{2.2486}$). Broken trend line refers to polyp number and solid line to colony area. Error bars are standard errors of the mean.

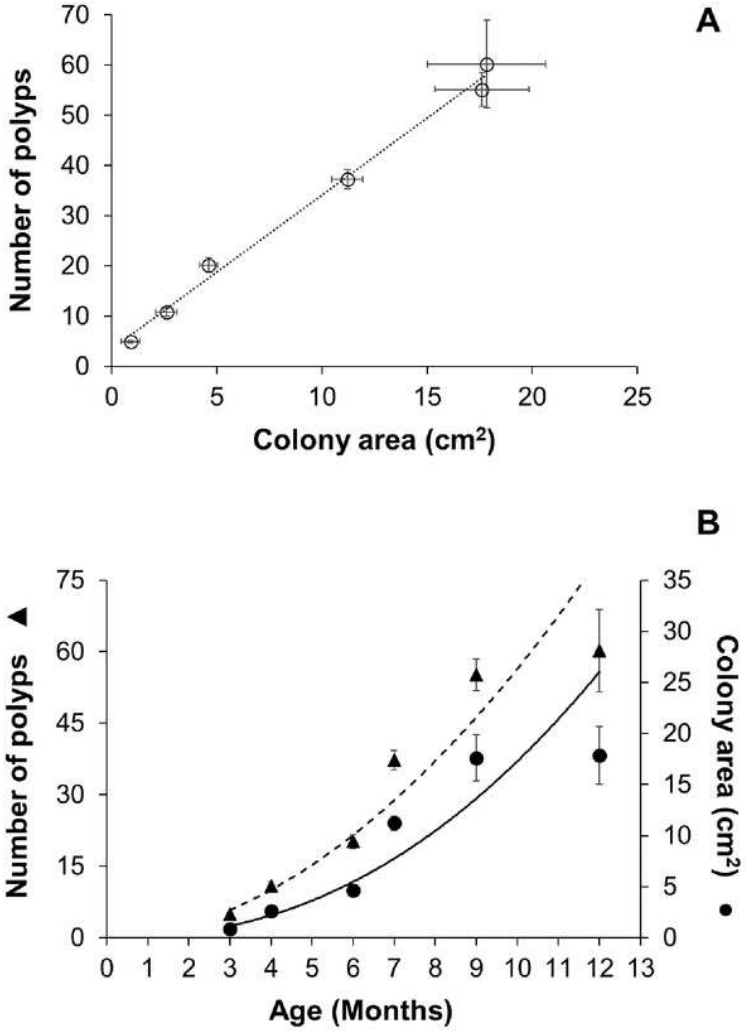
A
Number of polyps
Colony area (cm²)
0
10
20
30
40
50
60
70
0
5
10
15
20
25
B
Number of polyps
Colony area (cm²)
Age (Months)
0
15
30
45
60
75
0
5
10
15
20
25
30
35
0 1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3

Growth rates of two cohorts (2011 and 2012) of *Acropora palmata*.

The colonies were produced by crossing wild-caught gametes and culturing the embryos and larvae through settlement and grow out in an *ex-situ* nursery. Growth was measured while in the *ex-situ* nursery, after transfer to an *in-situ* nursery, and after outplanting onto Cuevones Reef. Error bars indicate standard errors of the mean.

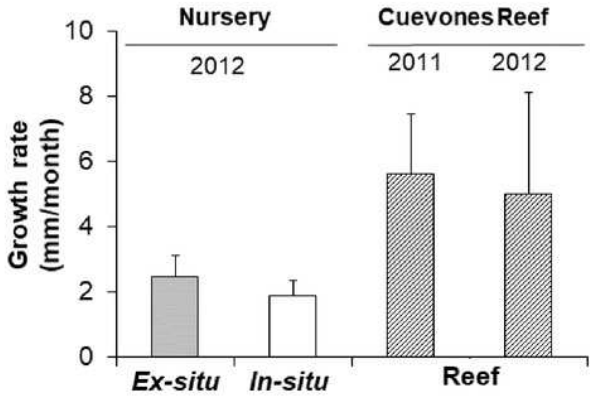
Nursery
Cuevones Reef
2012
2011
2012
Growth rate (mm/month)
10
8
6
4
2
0
Ex-situ
In-situ
Reef

Figure 4

Comparison of growth rates of F1 *Acropora palmata* colonies outplanted on Picudas Reef.

Growth was measured on the reef during two years after outplanting onto the reef. Error bars indicate standard errors of the mean.

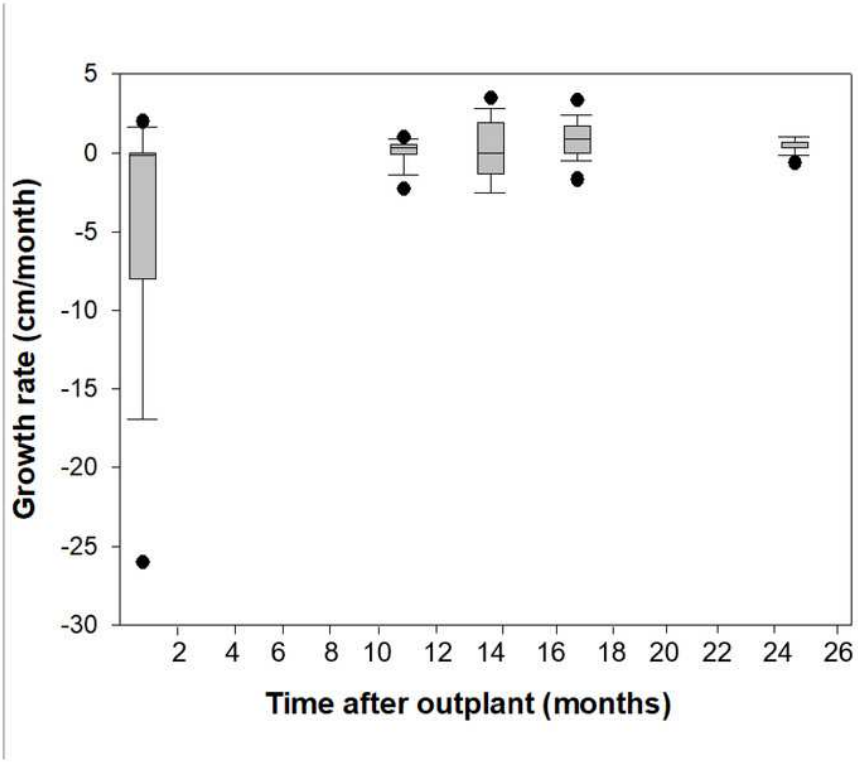
Growth rate (cm/month)
5
0
-5
-10
-15
-20
-25
-30
2
4
6
8
10
12
14
16
18
20
22
24
26
Time after outplant (months)

Figure 5

Reproductive capacity of *Acropora palmata* colonies produced in the laboratory and outplanted onto Cuevones Reef.

(A) *Acropora palmata* colony at 9 years old on Cuevones Reef. Scale bar = 10 cm. (B) Fragment of *Acropora palmata* colony showing developing oocytes (pink). Scale bar = 1 cm. (C) Cross sectional and (D) longitudinal views of developing oocytes (O) and spermaries (S) in decalcified coral polyp. Scale bar = 1 mm.

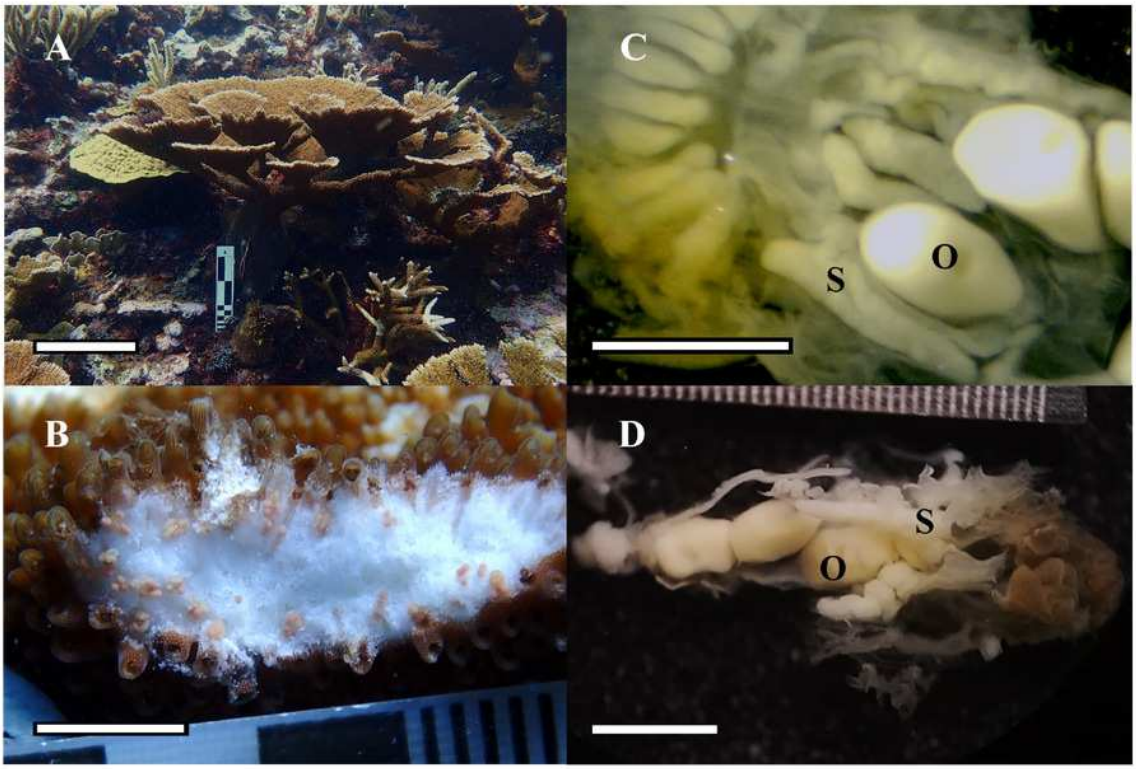

Figure 6

Spawning by the F1 generation of *Acropora palmata* colonies and their offspring.

(A) Spawning by an F1 generation *Acropora palmata* colony that was produced by collecting wild gametes, assisting in fertilization, culturing, and outplanting onto Picudas Reef. Scale bar = 10 cm. (B) Closeup of photo A to show setting where gamete bundles can be observed in the mouths of the polyps just prior to spawning. Scale bar = 5 cm. (C) F2 generation of 10-day-old recruits of *A. palmata* without symbionts and with skeleton clearly visible. Scale bar = 1 mm. (D) Five-month-old F2 generation *A. palmata* recruit in *ex-situ* culture. Scale bar = 1 mm. Photo credits: A and B: Esmeralda Pérez Cervantes, C: Sandra Mendoza Quiroz, D: Victoria Grosso.

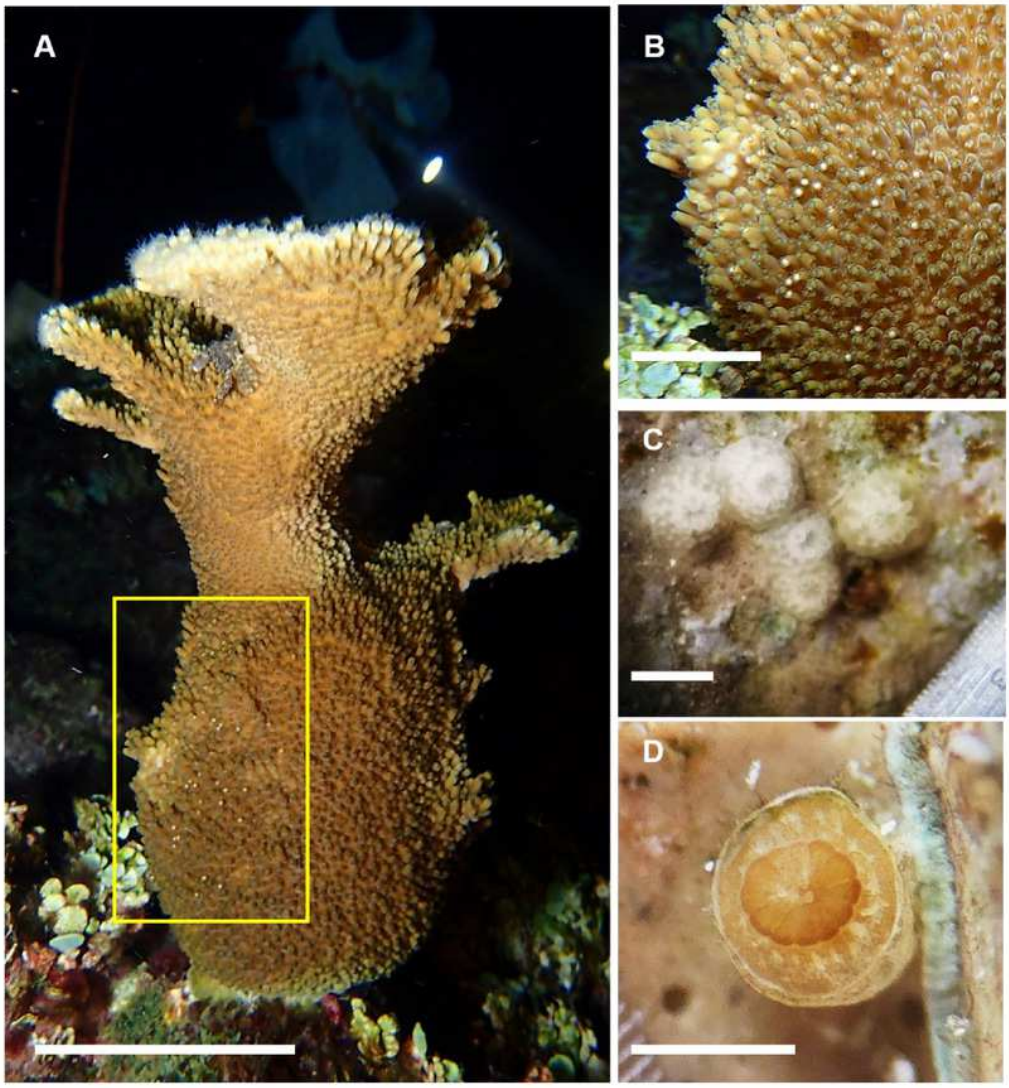
A
B
C
D

Table 1(on next page)

Survival of *Acropora palmata* cohorts.

The number of colonies of *Acropora palmata* produced in 2011 and 2012 that survived in e*x-situ* facilities, after transfer to an *in-situ* nursery and after outplanting onto Cuevones Reef.

Table 1 Survival of the 2011 and 2012 *Acropora palmata* cohorts in e*x-situ* facilities, after transfer to an *in-situ* nursery and after outplanting onto Cuevones Reef.

	2011				2012			
	Date	**Age**	**UNAM**	**XCARET**	**Date**	**Age**	**UNAM**	**XCARET**
Ex-situ	sep-11	1 mo	1155	-	dic-12	3 mo	-	393
	feb-12	6 mo	119	35	jun-13	9 mo	-	151
	may-12	9 mo	12	12	sep-13	12 mo	-	112
	aug-12	12 mo	12	12	nov-13	14 mo	-	107
	sep-13	25 mo	12	12	mar-14	18 mo	-	46
In-situ	apr-14	2.6 y	12	12	apr-14	1.6 y	18	18
	aug-14	3 y	12	12	sep-14	2 y	18	18
	feb-15	3.5 y	12	12	feb-15	2.4 y	18	18
Reef	feb-15	3.5 y	8	12	feb-15	2.5 y	5	4
	aug-17	6 y	8	12	aug-17	5 y	5	4
	aug-20	9 y	8	12	aug-20	8 y	5	4

Table 2(on next page)

Characteristics of the F1 generation *Acropora palmata* colonies of sexual origin that spawned in August 2022.

Size, age, and condition of *A. palmata* colonies as well as the volume of gametes (mL) are shown.

1

Cohort	ID Colony	Spawn Collection	Gamete volume (mL)	Length (cm)	Width (cm)	Height (cm)	Number of branches	Colony condition
2011	120	no	~50 boundles	25	20	30	3	Healthy
2012	324	yes	2	22	17	15	4	Healthy
2012	441	yes	2	40	35	45	7	Healthy
2012	125	yes	0.1	16	18	23	5	Healthy

2